R E M A R K S

Claims 2, 4, 6, 7, 9, and 14-22 are pending in this application. No new matter has been added by way of the above amendments. For example, claims 1, 3, 5, 8 and 10-13 have been canceled. New claim 16 is now present as the main independent claim in replacement of claim 1. The subject matter of new claim 16 is supported by the present specification at page 1, lines 12-14, page 4, lines 22-25, page 8, lines 8-14 and page 19, lines 20-21. Claims 2, 4, 6, 7, 9, 14 and 15 have been amended to be consistent with independent claim 16. New claim 17 is supported by the present specification at page 9, lines 14-18. New claim 18 is supported by the present specification at page 19, line 22 to page 20, line 4. New claim 19 is supported by the present specification at page 21, line 25 to page 22, line 4. New claim 20 is supported by the present specification at page 21, line 20 to page 22, line 4. New claims 21-23 are supported by the present specification at page 5, line 20 to page 6, line 1. Regarding the specification, applicants have amended the specification to correct some minor typographical errors and to provide a reference to Japanese priority application 323684/1997. Applicants have also provided a new Abstract of Disclosure, which corrects a minor

typographical error. Lastly, applicants have provided a new Title of the Invention, which is descriptive.

Accordingly, no new matter has been added. A marked up version showing changes made is attached hereto.

In view of the following remarks, Applicants respectfully request that the Examiner withdraw all rejections and allow the currently pending claims.

Issue relating to Priority

At page 2 of the outstanding Office Action, the Examiner notes that the application does not contain a sentence referencing priority document Japanese application No. 9-323684, filed on November 26, 1997 in Japan and Taiwan application No. 87119150 filed on November 19, 1998 in Taiwan. Applicants have added a reference to the Japanese priority document; however, Applicants have not referenced Taiwan application No. 87119150 due to the fact that no priority has been claimed to this Taiwanese application. Accordingly, no certified copy of the Taiwanese application is required either.

The Examiner is requested to acknowledge the above.

Issue relating to the Drawings

Applicants have amended the specification to reflect the fact the application contains at least one drawing executed in color.

Objections to the Specification

The Examiner has objected to the specification for the reasons recited at page 4 of the outstanding Office Action. Applicants respectfully traverse.

First, the Examiner has pointed out a typographical error in the Abstract of Disclosure. Applicants have amended the Abstract accordingly.

Second, the Examiner has requested that a new title, which is clearly indicative of the invention be added. The Examiner has graciously suggested a new title, however, Applicants do not believe that the suggested title is suitable due to the fact that the protein focused by the present invention is not α_{2u}-globulin. Thus, instead of adopting the Examiner's suggestion, Applicants have amended the title to read "Method for examining human kidney diseases by detecting the fatty acid binding protein".

Third, the Examiner has objected to the disclosure at page 25, line 17 for a minor typographical error. Applicants have made a suitable correction.

Fourth and lastly, the Examiner has objected to the use of trademarks in the application. Applicants have amended the specification to respect the proprietary nature of the marks.

In view of the above, applicants submit that all the objections to the specification are overcome. Reconsideration and withdrawal of these objections are requested.

Rejections under 35 U.S.C. §112, second paragraph

The Examiner has rejected the present claims under 35 U.S.C. § 112, second paragraph for the reasons recited at pages 5-6 of the outstanding Office Action. Applicants respectfully traverse.

First, the Examiner has rejected the use of "examining" as being vague and indefinite. Applicants respectfully submit that the use of the term "examining" is now avoided in the claims as amended. Thus, this rejection is moot. Reconsideration and withdrawal thereof are respectfully requested.

Second, the Examiner has rejected the utilization of the terms "liver-type", "muscle-type" and "kidney-type". Applicants respectfully traverse.

The term "fatty acid binding protein" does not necessarily refer to a protein that binds to fatty acid, but is a generic name for indicating a certain protein (or a group of proteins). As the "fatty acid binding protein" (hereinafter, referred to "FABP"), species of molecules are known, which are specifically distributed in tissues, respectively. These proteins have the ability to bind to fatty acids, and hence, it is assumed that these proteins belong to the same family. Such a family would have progressed from common ancestral genes. These species of molecules are designated as follows, respectively.

1) "liver-type FABP" (which is also designated as L-FABP, liver FABP type, or liver FABP)
2) "heart-muscle-type FABP" (which is also designated as H-FABP, heart-type FABP, heart FABP type, heart FABP, muscle-type FABP, or skeletal muscle FABP). It is known that the heart type-FABP (heart FABP) and muscle-type FABP (skeletal-muscle FABP) are identical, i.e., both are in the same species of molecules (cf.

Exhibit II, Veerkamp et al., Prog. Lipid Res., 34:17-52, 1995, page 22, lines 21-22.

3) "intestinal-type FABP" (which is also designated as I-FABP, intestinal FABP type, or intestinal FABP); [cf. Exhibit I: Maatman et al., Biochem. J., 288: 285-290, 1992, particularly see page 285, left column, lines 8-12 and Exhibit II: Veerkamp et al., Prog. Lipid Res., 34:17-52, 1995, particularly page 21, lines 25-26 and Table 3] (a copy of these exhibits is attached).

Accordingly, the terms "liver-type fatty acid binding protein", "heart-muscle-type FABP", etc. are the names indicating species of proteins.

Each species of molecules of FABPs is designated on the basis of the tissue of origin, in which the species were first discovered. For example, the "liver-type FABP" means a protein which species has first been found in liver tissue or a species identical therewith. The "heart-muscle-type FABP" means a protein which species has first been discovered in heart tissue or in skeletal muscle tissue or a species identical therewith.

It should, however, be noted that the indication of tissue in these terms does not necessarily mean that said species exists or is produced only in the indicated tissue. In fact, it

is known that two kinds of FABP exist in the human kidney, but one of them is identical with a fatty acid binding protein produced in the human liver which has been confirmed on the basis of the amino acid sequence, etc. (cf. Exhibit I, page 285, Abstract, lines 3-4, and page 287, left column, lines 28-29). The latter species of molecules is the "liver-type fatty acid binding protein".

Moreover, it has been confirmed that another species existing in the human kidney is the same species of molecule as that of the heart-type or muscle-type FABP (cf. Exhibit I, page 285, Abstract, lines 3-4, and page 287, right column, lines 1-3). This species of molecules is the "heart/muscle-type fatty acid binding protein".

As is clear from the above explanation, the terms the "liver-type fatty acid binding protein", "heart/muscle-type fatty acid binding protein" each refer to a species of molecule of a specific FABP, respectively, and hence, these terms are not vague.

Additionally, in scientific literatures, the "liver-type fatty acid binding protein" (or "fatty acid binding protein, liver-type") may occasionally be shown by a term "liver fatty acid binding protein", but both mean the same protein. However,

sometimes, the wording "**liver** fatty acid binding protein" is used for meaning a FABP protein that is produced or exists in the liver. Accordingly, in order to avoid any possible confusion, in the present invention, the wording "**liver-type** fatty acid binding protein" is used.

The Examiner's rejection with respect to "type" is therefore overcome. Reconsideration and withdrawal thereof are respectfully requested.

Third, the Examiner has rejected the use of the acronym GMB in claim 13. However, claim 13 has been deleted. Thus, this rejection is moot. Reconsideration and withdrawal thereof are respectfully requested.

Fourth, the Examiner has rejected the term "substantially" in claim 9. This term has been deleted from claim 9, thus, this rejection is moot. Reconsideration and withdrawal thereof are respectfully requested.

Fifth, and lastly, the Examiner asserts that claims 1-14 are incomplete for omitting essential steps. Applicants traverse. New claim 16 recites specific steps, thus, this rejection is overcome.

Applicants further point out that as explained above, the term "liver-type fatty acid binding protein" does not mean a

protein that binds to fatty acid, but means a species of molecules of specific proteins, and the characteristic point of the present invention is in the detection of the presence of said protein ("liver-type fatty acid binding protein") including a protein which does not bind to a fatty acid. It is not focused on whether or not the protein binds to fatty acid, in other words, it is not essential in the present invention to detect whether or not the protein binds to a fatty acid. Moreover, it is not a necessary step in the present invention to form a complex bound to fatty acid (i.e., a fatty acid binding complex). but a liver-type fatty acid complex is necessary.

Accordingly, applicants respectfully submit that the present claims satisfy the requirements of 35 U.S.C. § 112, second paragraph. The Examiner is therefore respectfully requested to withdraw these rejections.

Issues under 35 U.S.C. § 112, first paragraph

The Examiner has rejected the pending claims under 35 U.S.C. § 112, first paragraph for the reasons recited at pages 7 and 8 of the outstanding Office Action. Applicants respectfully traverse.

First, the Examiner asserts that the present claims lack sufficient steps. Applicants traverse and submit that claim 16 (which is now the main independent claim) recites specific steps for practicing the present method. Thus, this rejection is moot. Reconsideration and withdrawal thereof are requested.

Second, the Examiner asserts that the present specification is enabling for detecting the fatty acid binding protein α_{2U}-globulin, but not all fatty acid binding proteins and all kidney disease detection. Applicants traverse. Applicants respectfully submit that the "fatty acid binding protein" to be detected has been limited to "human" liver-type fatty acid binding protein. The method of detection of human L-FABP is well supported by the present specification, specifically Examples 1-5. As to the difference between L-FABP and α_{2U}-globulin discussed by the Examiner (Item 17 of the outstanding Office Action), Applicants submit that the Examiner is in error. The Examiner is referred to the distinction as discussed below under the heading Distinctions Between the Present Invention and Olson et al.

Accordingly, the present claims define subject matter which meets the requirements of 35 USC § 112, first paragraph. The Examiner is therefore requested to withdraw these rejections.

Issues under 35 U.S.C. § 102(b)

The Examiner has rejected claims 1, 2, 4-8, 10, 11 and 12 under 35 U.S.C. § 102(b) as being anticipated by Olson et al. Applicants respectfully traverse.

The present invention is concerned with a method for diagnosis or prognosis of kidney disease in a human, which is characteristic in detecting "liver-type fatty acid binding protein" (L-FABP) in a specimen collected from a human, as well as a reagent or kit for said diagnosis or prognosis. According to the method of the present invention, the prognosis, etc. of the kidney disease in a human can conveniently be practiced.

Distinctions Between the Present Invention and Olson et al.

Olson disclose the results of a comparison of urine protein between male rats and humans, and based on these results (that is, the protein content of urine was lower in human and the ratio of cationic protein to total protein was lower in human), it is suggested that there is no risk of hydrocarbon-induced nephropathy in human (cf. Olson et al., Abstract, lines 5-1 from the bottom).

Olson also disclose that high amounts of α_{2U}-globulin in male rats have some relation to susceptibility to Hyaline droplet nephropathy (cf. Olson et al., page 525, left column, 2nd paragraph).

Olson et al. investigated α_{2U}-globulin as the urine protein in rats, and α_1-microglobulin and α_1-acid glycoprotein as the α_{2U}-globulin related protein in humans, which are in the same family as the α_{2U}-globulin in a rat. However, it is known that α_{2U}-globulin is a protein specific in rats and does not appear in humans. Moreover, although α_{2U}-globulin, α_1-microglobulin and α_1-acid glycoprotein all belong to a protein family of "Lipocalines", they are entirely different species from L-FABP (liver-type fatty acid binding protein), which belongs to a family distinct from "Lipocalines". [cf. Exhibit I, page 285, left column, lines 21-29; Exhibit II, page 18, Table 1; and Exhibit III: Pervaiz et al., FASEB J., 1:209-214, 1987, particular page 209, Abstract, line 5-10, a copy of Ex. III is enclosed.]

Moreover, it is also known that α_{2U}-globulin existing in the kidneys of rats (which is also designated as renal-type FABP in rats) is entirely different from L-FABP existing in kidneys of

humans with respect to the manner of expression. That is, α_{2U}-globulin existing in kidneys of rats is first excreted in the liver and taken up by the kidney (through proximal tubules) (cf. Exhibit I, page 285, left column, lines 21-29). On the contrary, the L-FABP existing in the kidneys of humans is usually produced directly within the kidney cells, which is supported by detection of mRNA thereof in the kidney tissue (cf. Exhibit I, page 286, right column, lines 4-1 from the bottom).

As is evident from the above, the α_{2U}-globulin, α_1-microglobulin and α_1-acid glycoprotein all belong to a species of molecules which is an entirely different species from L-FABP. Further, α_{2U}-globulin in rats is entirely different from L-FABP in humans with respect to the manner of expression in the kidney.

Accordingly, the method of the present invention aiming at the L-FABP originating from a human kidney is clearly distinguished from Olson et al. Therefore, the present claims are not anticipated by Olson et al.

No distinction between claims.

Rejections under 35 U.S.C. § 103(a)

The Examiner has rejected claims 3 and 9 under 35 U.S.C. § 103(a) as being obvious over Olson et al. in view of Kimura et al.

The Examiner has also rejected claim 13 under 35 U.S.C. § 103(a) as being obvious over Olson et al. in view of Galaske et al.

The Examiner further rejected claims 14 and 15 under 35 U.S.C. § 103(a) as being obvious over Olson et al. in view Zuk et al.

Applicants respectfully traverse each of the above rejections.

Distinctions Between the Present Invention and the Cited Art

The Examiner has utilized the Olson et al. reference as the primary reference in each of the rejections under 35 USC § 103(a). As discussed above, the present invention is distinguishable from the Olson et al. reference. That is, the α_{2U}-globulin, α_1-microglobulin and α_1-acid glycoprotein disclosed by Olson all belong to a species of molecules which is entirely different from L-FABP of the present invention. Further, α_{2U}-globulin in rats is entirely different from L-FABP in humans

with respect to the manner of expression in the kidney. Thus, significant deficiencies exist in the primary reference of Olson. Even when the secondary references are combined with the primary reference, one does not arrive at the present invention as claimed. This will be further discussed below with respect to the specific claims rejected by the Examiner as obvious.

Claim 3 -Note that claim 3 was deleted.

Claim 9 - The invention of claim 9 relates to a method for diagnosis or prognosis of a kidney disease in humans where the antibody specifically binding to the liver-type FABP is an antibody that does not cross-react with a heart muscle-type FABP.

Claim 13 - The invention of claim 13 relates to the method where the kidney disease are anti-GMB nephritis model. Note that claim 13 was deleted.

Claims 14 and 15 - The invention of claims 14 and 15 is concerned with a reagent or kit for diagnosis of prognosis for a kidney disease.

The contents of the primary Olson et al. reference are explained above, and the contents of the secondary references are briefly as follows.

(i) Kimura et al. reference:

This reference is concerned with a fatty acid binding protein in the kidney of rats. Kimura disclose that in two fatty acid binding proteins, one protein of 14 kDa was identified as heart-type FABP which is localized in the distal tubules of the rat kidneys, and another protein of 15.5 kDa was identified as a modified protein of α_{2U}-globulin which is observed predominantly in the proximal tubules of the rat kidneys. Kimura further disclose that a protein of 14 kDa (being the same as heart-type FABP) was observed in female rat kidneys.

(ii) Galaske et al. reference:

This reference is concerned with an experimental model of anti-GMB nephritis. Galaske discloses preparation of the anti-GMB nephritis model, glomerular filtration of serum protein in the acute heterologus phase of the anti-GBM nephritis.

(iii) Zuk et al reference:

This reference is concerned with an assay for detecting organic materials (more particularly), immunoassay), and discloses reagents and kits used for the assay.

None of the secondary references cited by the Examiner disclose or even suggest liver-type FABP (L-FAPB) originated from human kidneys. Thus, even when the references are taken as a whole, the deficiencies of the primary Olson reference are not overcome. Thus, the Examiner has failed to provide a proper *prima facie* case of obviousness. Therefore, the rejection of the present claims is improper and should be withdrawn.

In summary, the present claims define subject matter which is patentable over the prior art of record. Thus, the Examiner is respectfully requested to withdraw these rejections and allow the currently pending claims.

Pursuant to 37 C.F.R. § 1.17 and 1.136(a), Applicants respectfully petition for a two (2) months extension of time for filing a reply in connection with the present application, and the required fee of $390.00 is attached hereto.

Should there be any outstanding matters that need to be resolved in the present application, the Examiner is respectfully requested to contact Craig A. McRobbie (Reg. No. 42,874) at the telephone number of the undersigned below.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees

required under 37 C.F.R. §§ 1.16 or 1.17; particularly, extension of time fees.

Respectfully submitted,

BIRCH, STEWART, KOLASCH & BIRCH, LLP

By #42,874
for Gerald M. Murphy, Jr., #28,977

P.O. Box 747
Falls Church, VA 22040-0747
(703) 205-8000

GMM/CAM:bmp
0020-4710P

Attachment: Version with Markings to Show Changes Made
Exhibits I, II and III

(Rev. 02/12/01)

VERSION WITH MARKINGS TO SHOW CHANGES MADE

IN THE TITLE:

A new title was added.

IN THE ABSTRACT OF THE DISCLOSURE:

The abstract was amended as follows:

--Method for examining kidney disease, which comprises detecting fatty acid binding protein derived from kidney tissues, which is present in specimen collected from mammal excluding Rodents. By the present method, it is possible to obtain test results, which may be very important information for diagnosis [of] or prognosis of kidney disease that has been very difficult in the past. Based on test results obtained by the present method, it may be possible to select a suitable method for treatment of kidney disease with taking into consideration risks such as the prognosis, etc. Besides, the present method can be applied to, in addition to the kidney disease samples, urine samples as well, so that the examination procedure can be simple and efficient.--

IN THE SPECIFICATION:

The specification was amended as follows:

The paragraph at page 1, lines 5-9 was amended as follows:

This application is a continuation-in-part application of PCT International Application No. PCT/JP98/05319 which has an international filing date of November 26, 1998 which designated the United States, and which claims priority to Japanese Application 9-323684/1997, which has a Japanese filing date of November 26, 1997 the entire contents of which are incorporated by reference.

The following new paragraph has been inserted at page 8, line 15 after "BRIEF DESCRIPTION OF THE DRAWINGS"

--The file of this patent contains at least one drawing executed in color. Copies of this patent with color drawing(s) will be provided by the Patent and Trademark Office upon request and payment of the necessary fee.--

The paragraph beginning at page 22, line 15 and ending at page 23, lines 1-18 was replaced with the following replacement paragraph:

--Thus, an anti-mouse L-FABP polyclonal antibody was prepared. The antigen, mouse L-FABP, was prepared according to the method disclosed in the literature (Takahashi et al., Eur. J. Biochem., vol. 136, p. 589-601, 1983), as follows. That is, to the excised liver from a mouse killed by bleeding was added a four-time volume of 30 mM Tris-HCl buffer (pH 8), and the mixture was treated by a polythoron-type homogenizer. The resultant was centrifuged at 8000 rpm for 15 minutes, and the supernatant thus obtained was further ultra-centrifuged at 100,000 x g for 90 minutes to give the cytoplasm fraction. The cytoplasm fraction was separated by gel filtration column ([Sephacryl] SEPHACRYL™ S-100HR, manufactured by Pharmacia Inc.), and the fractions exhibiting a fatty acid binding activity were collected, checking as an indicator a binding activity to ANS (1-anilinonaphthalene-8-sulfonic acid, manufactured by Polysciences, Inc.). The obtained fractions of a molecular weight of 10 to 20 kilodalton were combined and dialyzed against 10 mM Tris-HC1 buffer (pH 8.5), and then charged onto an anion exchange column (HiTrap Q, manufactured by Pharmacia Inc.) and eluted with a solvent of liner gradient to 500 mM NaCl, and the fractions exhibiting an ANS-binding activities were collected. Moreover, the resultant was further

separated by gel filtration column ([Sephacryl] SEPHACRYL™ S-100HR, manufactured by Pharmacia Inc.) in the same manner as above, and each fraction thus obtained was subjected to SDS-polyacrylamide gel electrophoresis, and the fraction showing a single band of about 14 kilodalton was collected go give a purified mouse L-FABP.--

The paragraph starting at page 25, line 12 and ending at page 26, line 1 was amended as follows:

--cDNA of human L-FABP was obtained by PCR (polymerase chain reaction) from the cDNA library derived from human liver (manufactured by CLONTECH Laboratories Inc., Cat # HL1115b Lot # 5621). An oligonucleotide of 23 to 27mers synthesized by a DNA synthesizer was used as a primer. The nucleotide [necleotide] sequence of the primer was designed based on the gene sequence of human L-FABP disclosed in the literature (Lowe et al., J. Biol. Chem., vol. 260, p. 3413-3417, 1985) and Gene Data Base (GENBANK Accession No. M10617), with adding a restriction enzyme recognition site for inserting an expression vector at the end of the primer. The obtained DNA fragment (about 420 base pairs) has a *Bam*HI recognition site before the initiation codon, and

the *Bam*HI recognition site after the termination codon, and encodes the desired full-length human L-FABP.--

The paragraph beginning at page 26, line 20 and ending at page 27, lines 1-15 was replaced with the following replacement paragraph:

--The obtained cells were broken by ultrasonic, and the cell extract was dialyzed against 5 mM Tris-HCl buffer (pH 8.5). The resultant was separated by anion exchange column (RESOURSE Q 6 ml, manufactured by Pharmacia, Inc.), eluted with a solvent of liner gradient to 300 mM NaCl, and the fraction showing ANS-binding activity was collected. The fraction was concentrated by ultra filtration with Centriprep (manufactured by AMICON LTD.), and separated by gel filtration column ([Superdex] SUPERDEX™ 75pg, manufactured by Pharmacia Inc.), and the fraction showing ANS-binding activity was collected to give a human L-FABP fusion protein. To the human L-FABP fusion protein thus obtained was added Factor Xa (manufactured by New England Biolabs Inc.) in 1/100 weight, and the mixture was reacted at room temperature overnight for restriction degradation. The reaction solution after the enzyme treatment was separated again by gel filtration, and the fraction of about 14 kilodalton showing

ANS-binding activity was collected to give a recombinant human L-FABP. The obtained purified protein was subjected to SDS-polyacrylamide gel electrophoresis, and subjected to silver staining, from which only one band was confirmed.--

IN THE CLAIMS:

Claims 1, 3, 5, 8, and 10-13 were cancelled.

The claims were amended as follows:

Claim 2. (Amended) The [examination] method according to claim 16 [claim 1], wherein the liver-type fatty acid binding protein [being] is (derived) from kidney tissue [is a liver-type fatty acid binding protein].

Claim 4. (Amended) The [examination] method according to claim 16 [claim 1], wherein the specimen is kidney tissue or urine.

Claim 6. (Amended) The [examination] method according to claim 16 [claim 1], which further comprises a [process for] step of comparing the test result with that of a control specimen,

said control specimen being collected from [an animal] a human having normal kidney tissue, or collected from a human having the same kidney disease but showing different symptoms or different progress.

Claim 7. (Amended) The [examination] method according to claim 16 [claim 1], wherein the detection of the liver type fatty acid binding protein in step (b) is carried out by using an antibody specifically binding to said liver-type fatty acid binding protein.

Claim 9. (Amended) The [examination] method according to claim 7 [claim 8], wherein the antibody specifically binding to the liver-type fatty acid binding protein is an antibody that [substantially] does not cross-react with a heart muscle-type fatty acid binding protein.

Claim 14. (Amended) A reagent or kit for diagnosis or prognosis [examination], which is used in the [examination] method according to any one of claims 16, 2, 4, 6-7, 9, and 17-23 [1-13].

Claim 15. (Amended) The reagent or kit for diagnosis or prognosis [examination] according to claim 14, which contains an antibody specifically binding to [a] liver-type fatty acid binding protein.

Claims 16-23 were newly added.

Biochem. J. (1992) 288, 285–290 (Printed in Great Britain)

Molecular identification of the liver- and the heart-type fatty acid-binding proteins in human and rat kidney

Use of the reverse transcriptase polymerase chain reaction

Ronald G. H. J. MAATMAN, Els M. A. VAN DE WESTERLO, Toin H. M. S. M. VAN KUPPEVELT and Jacques H. VEERKAMP*

Department of Biochemistry, University of Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands

The cDNAs of two types of fatty acid-binding protein (FABP) present in human kidney, previously described as types A and B, were isolated using reverse transcriptase-PCR (RT-PCR) with human kidney mRNA and various sets of primers. The cDNA fragments were cloned and sequenced. Renal FABP type A and B cDNAs appeared to be completely identical to human liver- and heart-type FABP cDNAs respectively. In the second part of this study we demonstrated the presence of liver-type FABP in rat kidney by chromatography, e.l.i.s.a. and immunocytochemistry. The ratio and cellular distribution of the two FABP types varies markedly in human and rat kidney. Using RT-PCR we were also able to prepare and identify liver- and heart-type FABP cDNAs with mRNA from both male and female rat kidney.

INTRODUCTION

In mammalian cells, fatty acids are important molecules for energy delivery and for synthesis of membrane lipids and lipid mediators. Fatty acids are metabolized in mitochondria, peroxisomes and on the endoplasmic reticulum. The transport of fatty acids from the plasma membrane to these cellular organelles is believed to be performed by fatty acid-binding proteins (FABPs) [1–3]. On the basis of their primary structure at least five FABPs, with sequence similarities of 25–65%, have been identified [3]. These FABPs have been named liver, heart, intestinal, adipocyte and myelin FABPs, after the tissue from which they were initially isolated. The existence of different FABP types suggests a type-specific function. The presence of more than one FABP type in a tissue, e.g. intestine [4], stomach [5] and kidney [6,7], supports this hypothesis. Since the first isolation of an FABP cDNA, the rat liver FABP cDNA [8], cDNAs of all five FABP types have been obtained [9–16].

In rat kidney two FABP types have been demonstrated. One was identified as heart-type FABP, based on biochemical and immunological characterization [8,17,18] and mRNA detection [11,19]. The second type was biochemically and immunologically different from heart- or liver-type FABP and was named renal-specific FABP [6,18,20]. Amino acid sequencing, however, demonstrated that this renal-type FABP was identical with α_{2U}-globulin [18,21]. The latter protein is a secretory protein of the liver and undergoes endocytotic uptake into the proximal tubules [22]. The α_{2U}-globulin molecule is structurally not an FABP, but is more closely related to the lipocalins such as lactoglobulin and serum retinol-binding protein [23,24]. Recently we isolated two FABP types from human kidney; one (type A) showed similarity with human liver FABP and the other (type B) was similar to human heart FABP [7]. However, cDNA analysis has yet to prove the exact identity of both human kidney FABPs.

In the present study we describe the preparation and identification of the two human renal FABP cDNA types by reverse transcriptase-PCR (RT-PCR). We also applied this PCR technique to investigate the FABP types of rat kidney, since we obtained evidence indicating the presence of liver-type FABP in this tissue.

MATERIALS AND METHODS

Materials

Moloney murine leukaemia virus (MMuLV) RNAase H⁻ RT was obtained from Bethesda Research Laboratories, Life Technologies, Gaithersburg, MD, U.S.A.; recombinant *Taq* DNA polymerase Amplitaq was from Perkin–Elmer Cetus, Norwalk, CT, U.S.A.; Sequenase version 2.0 was from United States Biochemical, Cleveland, OH, U.S.A.; synthetic oligonucleotide primers were from Pharmacia, Uppsala, Sweden; wheat germ extract, L-[^{35}S]methionine, [α-^{35}S]dATP (15 mCi/ml) and [α-^{32}P]dATP (10 mCi/ml) were from Amersham; SP6-RNA polymerase, RNasin and pGEM-5 Zf[+] were from Promega Corporation, Madison, NY, U.S.A.; peroxidase-conjugated goat anti-(rabbit IgG) was from Tago, Burlingama, CA, U.S.A.; rabbit peroxidase–anti-peroxidase couples were from Dakopattis, Glostrup, Denmark; goat anti-(Tamm–Horsfall glycoprotein) was from Cappel, West Chester, PA, U.S.A.

Human kidney tissue was obtained after nephrectomy. Rat kidneys were from 12-week-old Wistar rats.

RNA isolation and blot hybridization

Total RNA was extracted with LiCl/urea by a modification of the procedure of Auffray & Rougeon [25]. Kidney tissue (1–2 g) was homogenized in a mixture of 3 M-LiCl/6 M-urea/20 mM-sodium acetate (pH 5.2)/heparin (2 mg/ml) and left overnight at 0 °C. The RNA precipitate was pelleted by centrifugation for 15 min at 16000 *g* and washed once with 4 M-LiCl/8 M-urea. After centrifugation for 15 min at 16000 *g* the pellet was dissolved in 50 mM-sodium acetate (pH 5.2)/0.2% SDS/2 mM-EDTA and extracted successively with phenol, phenol/chloroform and chloroform. Subsequently the RNA was precipitated with ethanol and stored at −20 °C. Poly(A)$^+$ RNA was selected on oligo(dT)–cellulose [26].

For Northern blot analysis, RNA was electrophoresed on 1% agarose gels in the presence of formaldehyde. Prior to electrophoresis, ethidium bromide was added to the RNA samples in order to allow visualization of the rRNAs in the gel. In this way it was ascertained that the amounts of RNA in the different lanes were approximately the same. Following electrophoresis the

Abbreviations used: FABP, fatty acid-binding protein; RT-PCR, reverse transcriptase PCR; MMuLV, Moloney murine leukaemia virus.

* To whom correspondence should be addressed.

Exhibit II (II)

Pergamon

0163-7827(94)00005-0

Prog. Lipid Res. Vol. 34, No. 1, pp. 17–52, 1995

0163-7827/95/$29.00

CYTOPLASMIC FATTY ACID-BINDING PROTEINS: THEIR STRUCTURE AND GENES

Jacques H. Veerkamp and Ronald G. H. J. Maatman

Department of Biochemistry, University of Nijmegen, Trigon Bldg, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands

CONTENTS

I. Introduction 17
II. Fatty acid uptake and transport and fatty acid-binding proteins 18
III. Distribution and structure of cytoplasmic fatty acid-binding proteins (FABPs) 21
A. Distribution 21
B. Primary structure 22
C. Tertiary structure 24
IV. Gene structure and regulation of expression 27
A. Adipocyte FABP gene 28
B. Myelin FABP gene 30
C. Heart FABP gene 30
D. Epidermal FABP gene 31
E. Intestinal FABP gene 32
F. Liver FABP gene 34
G. CRBP I and II genes 38
H. CRABP I and II genes 40
V. Expression of members of the FABP family in heterologous systems 42
VI. Site-directed mutagenesis 42
Acknowledgements 45
References 45

I. INTRODUCTION

Lipid-binding, -transfer or -exchange proteins are present in intra- and extracellular fluids of all organisms. They play a role in the transport or targeting of lipids in the cell or in the plasma, but may also interact directly or indirectly by modulation of the free ligand concentration with various cellular processes. Some lipid-binding proteins are rather specific, others bind various hydrophobic ligands, e.g. serum albumin, non-specific lipid transfer protein, liver fatty acid-binding protein (FABP). Lipid-binding molecules belong to several unrelated families of proteins and many molecules have not been characterized well. The structure of three families of lipid-binding proteins has been established (Table 1). Many of these proteins bind fatty acids as their main ligand, but also proteins with a quite different structure have affinity to fatty acids (Table 2). Before we come to the main subject of this review, the cytoplasmic fatty acid-binding proteins (FABPs) and the other members of the FABP family, we will first shortly discuss other proteins which bind fatty acids.

Albumin is the main transporter of free fatty acids in the blood,[318] but in the fetal blood α-fetoprotein and fetuin are also involved in fatty acid transport.[342] Serum vitamin D-binding protein has a low affinity for fatty acids.[53] The structure of the fatty acid-binding sites of albumin[319] and human α-fetoprotein[236] have been described. Lactoglobulin is the only member of the lipocalins (Table 1) which has a rather high affinity for fatty acids,[320,264] but also binds retinol.[264] Heat-shock protein (72 kDa) contains 2 molecules of nonesterified palmitic and oleic acid each per isolated dimer.[109] Glutathione *S*-transferases bind fatty acid in their regulatory domain II at the C-terminal side.[184,235] Other proteins which bind fatty acids are the membrane fatty acid-binding proteins found in *Escherichia coli*[37] and in the plasma membrane of various mammalian cell types as adipocytes, enterocytes, hepatocytes and myocytes.[262,315] We will discuss these proteins below.

TABLE 1. Members and Characteristics of Three Families of Lipid-Binding Proteins

I	II (Lipocalins)	III
Albumin	β-Lactoglobulin	FABPs (7 types)
α-Fetoprotein	Serum retinol BP	CRBPs (2 types)
Vitamin D BP	Bilin BP (insect)	CRABPs (2 types)
	α_1 Microglobulin	
	Apoliproprotein D	
	Odorant BP	
	α_{2U} Globulin	
50–70 kDa	18–20 kDa	12–15 kDa
Double loops	β-Barrel of 8 strands	β-Clam of 10 strands
Extracellular	Extracellular	Intracellular

The extracellular lipocalins show similar architectural principles as the family of intracellular binding proteins of fatty acids, retinol and retinoic acid (Table 1). Proteins of both families possess a β-barrel structure of eight and ten strands, respectively, with the first strand shared by two orthogonal sheets.[100,144,255,285] The integrity of the barrel is retained upon removal of the ligand. The lipocalins act as extracellular binding proteins for different hydrophobic ligands, among which retinol is bound by various members. Recently an enzyme, prostaglandin D synthase, was identified as a member of the lipocalin family of hydrophobic molecule transporters.[220]

The structure of intracellular glycolipid-, phospholipid-, cholesterol- and many other lipid-binding proteins have no resemblance to that of the FABPs. Intracellularly, also an acyl-CoA binding protein is present.[168,169] This molecule has a completely different structure, although the acyl chain is also bound like in FABP in a bent conformation near non-polar residues in the interior of the protein.[170] This acyl-CoA binding protein does not bind fatty acids, but has a very high affinity to acyl-CoA esters[266,267,275] and is involved in their intracellular metabolism.[169,267]

In this review we will shortly describe the membrane fatty acid-binding proteins, but the (cytoplasmic) FABPs form the main subject. Since we and others previously considered many structural and possible functional aspects of these latter molecules,[17,19,21,97–99,102,145,208,249,350,374,375] we discuss especially recent data on their gene structure and on regulation of their gene expression. Fatty acids and FABPs appear to be directly or indirectly involved in modulation of several cellular processes including gene expression.[375] The latter point will also be taken into consideration in this review. For comparison we include some related data on the other members of the FABP family, the cellular retinol-binding proteins (CRBP I and II) and the cellular retinoic acid-binding proteins (CRABP I and II).

II. FATTY ACID UPTAKE AND TRANSPORT AND FATTY ACID-BINDING PROTEINS

The mechanism by which fatty acids traverse the plasma membrane has already been a controversial point for many years.[249,374] To understand fatty acid uptake, it is essential

TABLE 2. Proteins with a High Affinity for Fatty Acids

Protein	*M* (kDa)	Other ligands	Number high binding sites	Occurrence
Albumin	68	Bilirubin, drugs	2–6	Plasma
α-Fetoprotein	70	Oestrogen	1	Fetal plasma and tissues
Fetuin	49	?	1	Fetal plasma
Vitamin D-BP	53	Vitamin D	1	Plasma
β-Lactoglobulin	35	no	1	Milk
Heat-shock protein	71	?	2	Intracellular
Plasma membrane FABP	40–85	?	1	Cellular
Cytoplasmic FABPs	15	No—various	1–2	Intracellular
Glutathione *S*-transferase	47	Bilirubin, heme, bile acid	1	Intracellular

to consider all potentially limiting steps and their implications for the process.[388] Diffusion and dissociation from albumin may limit fatty acid uptake at physiologic albumin concentration.[90,363,390] The presence of albumin receptors and their relation with fatty acid uptake is equivocal in hepatocytes.[277,388,389] but they may exist in endothelium[7] and cardiomyocytes.[260] Receptor-mediated transcytosis of albumin may play a role in the modulation of the amount of fatty acids that are delivered to the target cells and tissues.[7] Evidence for a dual-receptor model was reported for the fatty acid uptake by resting and activated lymphocytes.[367] Albumin- and alpha-foetoprotein receptors as well as plasma membrane fatty acid-binding protein would be involved.

Fatty acid transport across the plasma membrane of various cell types has been considered as a passive process by simple diffusion and lipid partition.[49,70,239,274] This transport is followed by binding to the cytoplasmic FABP and transfer to cellular organelles[249,374] (Fig. 1) or further flow along internal membranes.[302] Native fatty acids and also most covalently labeled fatty acids move rapidly across a phospholipid bilayer via the unionized form[149,150] in contrast to 12-(9-anthroyloxy)stearic acid.[165,334] The slow flip–flop rate of this fatty acid has been used to postulate the necessity of proteins for transport of fatty acids across membranes.[165] Other investigations gave also evidence for a plasma membrane fatty acid-binding protein- or carrier-mediated uptake. The fast flip–flop of unchanged fatty acids may, however, provide a simple and energy-dependent mechanism for their entry into hepatocytes, myocytes and adipocytes. In biological membranes a membrane fatty acid-binding protein may be required to sequester the fatty acids to the phospholipid bilayer, where they can undergo flip–flop.[249,374] A transmembrane transporter protein as postulated in the mitochondrial inner membrane[311] could have a function in transfer of ionized fatty acid across the membrane.[150]

In *Escherichia coli* long-chain fatty acids require for their transport across the outer membrane the presence of the FadL protein.[57] The high fatty acid-binding affinity of this

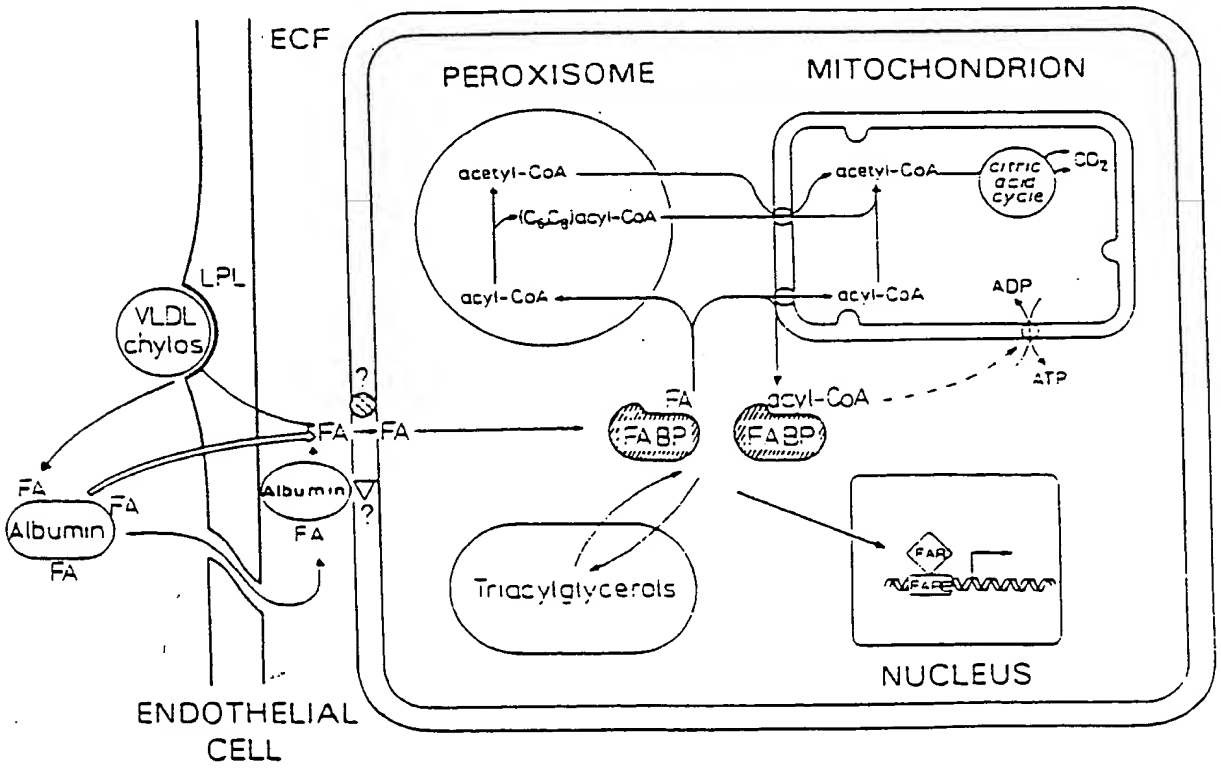

Fig. 1. Schematic representation of the fatty acid transfer from blood to cellular organelles and systems of lipid synthesis. Abbreviations: FA, fatty acid; VLDL, very low density lipoproteins; chylos, chylomicrons; ECF, extracellular fluid; FAR, putative FA receptor; FARE, putative FA receptor element.

protein could be established by photo-affinity labeling.[204] Studies on mutants of FadL demonstrated that the amino acids Phe^{448}, Pro^{428}, Val^{410} and Ser^{397} of the carboxyl region of this protein are involved in the binding and/or transport of fatty acid.[172,173] The ways of transport of fatty acid through the periplasmic space and across the inner membrane of the bacterium have not been defined. No evidence was found for the presence of a fatty acid-binding protein in the inner membrane of the cell envelope.[204] Recruitment of acyl-CoA synthetase to the inner membrane resulted in a 10-fold increase in the rate of fatty acid transporter.[205] This may indicate that the membrane-associated pool of fatty acids is the direct substrate for the enzyme.

The involvement of membrane fatty acid-binding proteins in fatty acid uptake into mammalian cells is based on action of inhibitors and proteases, on binding and kinetic data, on the isolation of these proteins and on the inhibition of fatty acid uptake by an antibody against such a protein.[262,315,316] The identity of these proteins is, however, rather unclear. Proteins with a molecular mass of 40–43 kDa were isolated by affinity chromatography from rat cardiac myocytes,[316,336] adipocytes,[301] jejunal microvillous membranes,[338] and liver plasma membranes.[339] The proteins appeared to be immunologically similar with a polyclonal antibody against the liver protein,[316] but not with a monoclonal antibody.[77] Antibodies against the protein from liver plasma membranes inhibited fatty acid uptake into hepatocytes, adipocytes and cardiac myocytes,[316,336,407] but not into keratinocytes in which a fatty acid uptake mechanism was found with preference for linoleic acid.[300] The surface expression of the protein is virtually identical in hepatocytes of male and female rats.[317]

The plasma membrane fatty acid-binding protein and the mitochondrial isoenzyme of aspartate aminotransferase from rat liver appeared to be related proteins,[29,341] but this was disputed.[337] Recently it was reported that fibroblasts transfected with an aspartate aminotransferase cDNA express both plasma membrane fatty acid-binding protein and saturable fatty acid uptake.[134] The 43-kDa plasma membrane fatty acid-binding protein was only expressed in differentiated adipocytes.[407] Other groups identified a plasma membrane fatty acid-binding protein of 22 kDa by photoaffinity labeling, both in undifferentiated fibroblasts and differentiated adipocytes.[359] Labeling with sulfo-*N*-succinimidyl oleate gave a plasma membrane protein with an apparent size of 85 kDa[114] in accordance with previous permeation studies.[2] Recently, the cDNA and the deduced protein structure of this rat adipocyte membrane protein implicated in fatty acid binding and/or transport were reported.[3] The sequence of the 472-amino acid (52.5 kDa) protein predicted two transmembrane segments and 10 potential N-linked glycosylation sites. The protein sequence is 85% similar with that of the glycoprotein IV (CD36) of platelets. Northern blots probed with the cDNA showed transcripts in differentiated adipocytes, heart, intestine, muscle and testis, but not in preadipocytes, liver and kidney.[3] Studies on fatty acid uptake in adipocytes with fluorescence microscopy indicated that a large fraction of transport is mediated by protein.[335] Treating adipocytes with the membrane-impermeable reagent 4,4′-diisothiocyanostilbene-2,2′-disulfonate inhibited more than 50% of the long-chain fatty acid transport. The role of the plasma membrane fatty acid-binding proteins in fatty acid uptake needs further evidence. Possibly they are necessary for uptake of ionized fatty acids and at low fatty acid concentrations for sequestering. They do not bind bilirubin, sulfobromophtalein, taurocholate, phosphatidylcholine or cholesteroloeate.[338,339] Nothing more is known about their ligand specificity and their tertiary structure is completely unknown.

Transport of fatty acids across subcellular membranes has not been studied except for mitochondrial membranes. Activation and formation of acylcarnitine esters on the outer membrane are followed by translocation of the acylcarnitine through the inner membrane.[265] A specific translocase is involved in this latter step.[219]

The function of cytoplasmic FABP in fatty acid uptake was theoretically discussed for hepatocytes[239,355] and cardiomyocytes.[380] The fatty acid distribution between blood and cytoplasm may be, similar to that of retinol, dependent on the distribution between albumin and FABP. Intracellular levels of retinol are regulated by the level of CRBP and

retinol distribution between serum retinol-binding protein and CRBP is at equilibrium.[238] Evidence for the involvement of FABP in fatty acid uptake on basis of physiological data and results with inhibitors of fatty acid binding to FABP is equivocal,[374] but recently FABPs of Hep G2 cells, primary rat hepatocytes and differentiated 3T3 adipocytes were labeled in a time- and temperature-dependent fashion at the uptake of a photoactivable radioiodinated fatty acid analogue.[328,359,381,382] Transport of fatty acids from and to natural and model membranes has been observed for different types of FABPs.[63,163,164,209,253,330–332,397] The transfer function of FABP was also demonstrated in a model cytosol system.[326] The surface charge on the FABP and on the membrane of the cellular organelles may influence the transfer rate from FABP to membrane.[119,397]

With laser photobleaching the intracellular transport of a fluorescent fatty acid analogue was characterized in cultured hepatocytes.[192] The cytoplasmic diffusion rate and the fraction of cellular fluorescent fatty acid in aqueous cytoplasm was larger in female than male cells corresponding with their difference in FABP content.[23,152,240,248] The investigators suggested that FABP and other cellular binding proteins may enhance the diffusive flux of their ligand by reducing membrane binding.[192]

III. DISTRIBUTION AND STRUCTURE OF CYTOPLASMIC FATTY ACID-BINDING PROTEINS (FABPs)

A. *Distribution*

FABPs have been isolated from cytosols of tissues of vertebrates and invertebrates by various procedures, including gel filtration, ion-exchange and affinity chromatography, precipitation with salt or organic solvents, and preparative electrophoresis.[249] The purification methods had to be adapted for different tissues, not only due to different contaminating proteins. Tissue-specific FABP types appeared to exist, which were named after the first tissue of isolation. At the moment at least seven FABP types have been established on base of amino acid and/or cDNA sequence (Table 3). They show a characteristic tissue and cellular distribution. The presence of a certain FABP type is mostly established by isolation of the protein or by Western and/or Northern blots, but in some tissues the immunochemical or immunohistochemical analyses need additional evidence.

In some tissues a certain FABP type is limited to specific cell species, e.g. liver FABP to hepatocytes.[20,89,130,346,347] In other tissues more FABP types are present in different or similar cell types. Four FABP types are expressed in the stomach, depending on the cell type and the developmental stage.[4,129] The liver and heart FABP types are present in the kidney, but at different locations.[194,195] In intestine both the liver and intestinal FABP types are found in enterocytes of jejunum and ileum, but in colonocytes only the liver FABP

TABLE 3. Tissue Occurrence of FABP Types*

Liver type‡	Liver,† intestine,† kidney,† stomach
Intestinal type	Intestine,† stomach
Heart type	Heart,† kidney,† skeletal muscle,† aorta,† Adrenals,† placenta,† brain,† testes, ovary, Lung, mammary gland, stomach
Adipocyte type§	Adipose tissue†
Myelin type‖	Peripheral nervous system†
Ileal type¶	Intestine,† ovary, adrenals, stomach
Epidermal type	Skin†

*The indication of a FABP type in a tissue does not mean its presence in all cell types of that tissue; the FABP type may be limited to specific cells or may be present at certain developmental stages.

†Evidence was obtained both by protein and mRNA analysis.

‡Previously termed Z-protein or aminoazo dye-binding protein A or wrongly sterol carrier protein.

§Also named adipocyte lipid-binding protein or initially p422 or aP_2.

‖Originally named myelin P_2 protein.

¶Also named ileal lipid-binding protein and originally gastrotropin.

type.[68,281,287,306,347,349] The ileal type[95,151,287] is not only present in the ileum, but also in the ovary and adrenal gland and in surface mucous cells of the stomach of the rat.[4] The heart FABP type is the most general one (Table 3). Besides heart and skeletal muscle many other tissues contain this protein.[69,248,373,384] The intestinal, adipocyte,[26,31,207] myelin[118,223] and epidermal[198,308,309] FABP types are limited to one tissue or organ system. More information on the cellular and topographical distribution of the FABP types is given in the section on the FABP genes (Section IV).

Immunoelectron microscopy and analysis of isolated cell fractions indicated in most cases the presence of FABP in the cytoplasmic matrix outside the organelles.[249,374] Some investigators observed heart FABP in the mitochondria of rat heart[92] and bovine heart.[43,366] Liver FABP was never detected in mitochondria.[44,89,248] The ileal FABP type was detected in the nuclear matrix of ileal enterocytes.[4] Liver FABP was observed in the nucleus of bovine[44] and rat[89] hepatocytes and heart FABP in the nuclei of bovine heart[43] and locust flight muscle.[116]

B. *Primary Structure*

The family of FABPs and related proteins contains at least 11 members, the seven FABP types, CRBP I and II and CRABP I and II (Table 4). The proteins contain 126–137 amino acids and show 38–70% similarity of amino acid sequence.[374,375] The aligned amino acid sequences are given in Fig. 2. These alignments show the large similarity between the heart, myelin, adipocyte and epidermal FABP types and the marked gap in the C-terminal part of the liver FABP and ileal FABP type. Human heart and skeletal muscle FABP appeared to be identical proteins.[254] CRBPs and CRABPs show 20–45% similarity with the FABPs. The similarity is 56% between rat CRBP I and II[181] and 74% between mouse CRABP I and II[96] and between human CRABP I and CRABP II.[9] The FABP types differ considerably in their surface charge. The heart FABP type has an isoelectric point of about 5.0, the liver and epidermal FABP types of about 6.0 and the adipocyte and myelin FABP types are basic proteins (p*I* values of 7.6–9.0).

Table 4 gives some characteristics of the amino acid composition and sequence of the members of the FABP family. The absence of tryptophan and cysteine from liver and intestinal FABP type, respectively, are peculiar. The large similarity of heart, adipocyte, myelin and epidermal FABP types (60–70%) is reflected in the similar amino acids on essential positions for fatty acid binding (see IIIC). These latter FABP types and CRBP I and II contain a protein tyrosine kinase recognition sequence before Tyr[19]. The extent of phosphorylation of FABP is, however, very slight under various conditions *in vitro* and in 3T3-L1 adipocytes, rat cardiomyocytes and mammary epithelial cells.[30,50,51,124,231,232] The buried position of Tyr[19] in the FABP molecule does not allow kinase action and conformational changes would be necessary.[52,72] Phosphorylation of Tyr[19] seems, however,

TABLE 4. Characteristics of Members of the FABP Family

Member	Phe "17"	Phe "57"	Arg "106"	Arg "126"	Tyr "128"	Trp	Cys
Liver FABP	14	ser	thr	122	ser	—	68
Intestinal FABP	17	57	106	126	phe	6, 82	—
Heart FABP	16	55	106	126	128	8, 97	124
Adipocyte FABP	16	55	106	126	128	8, 97	117
Myelin FABP	16	55	106	126	128	8, 97	117, 124
Epidermal FABP	18	leu	108	128	130	10, 99	119, 126
Ileal FABP	17	gly	ala	123	ser	48	68
CRBP I	16	57	gln	gln	phe	8, 88, 106, 109	95, 126
CRBP II	16	57	gln	gln	phe	8, 88, 106, 109	121, 125
CRABP I	15	val	111	131	133	87, 109	95, 129
CRABP II	15	val	111	132	134	87, 109	95, 130

All differences are derived from human proteins, except for ileal FABP and CRBP II from pig and rat, respectively. Methionine on position 1 was not taken into consideration for numbering. Positions between quotation marks are compared with intestinal FABP and refer to amino acid residues involved in fatty acid binding or portal region.

```
                1
 L-FABP  MS....FSGK YQLQSQENFE AFMKAIGLPE EL..IQKGKD IKGVSEIVQN 43
 I-FABP  MA....FDST WKVDRSENYD KFMEKMGVNI VKRKLAAHDN LK..LTITQE 43
 H-FABP  MVDA..FLGT WKLVDSKNFD DYMKSLGVGF ATRQVASMT. .KPTTIIEKN 45
My-FABP  .SNK..FLGT WKLVSSENFD DYMKALGVGL ATRKLGNLA. .KPTVIISKK 45
 A-FABP  MCDA..FVGT WKLVSSENFD DYMKEVGVGF ATRKVAGMA. .KPNMIISVN 45
 E-FABP  MATVQQLEGR WRLVDSKGFD EYMKELGVGI ALRKMGAMA. .KPDCIITCD 47
Il-FABP  MA....FTGK YEIESEKNYD EFMKRLALPS DA..IDKARN LKIISEVKQD 43
  CRBPI  MPVD..FTGY WKMLVNENFE EYLRALDVNV ALRKIANLL. .KPDKEIVQD 45
 CRBPII  MTKD..QNGT WEMESNENFE GYMKALDIDF ATRKIAVRL. .TQTKITVQD 45
 CRABPI  MPN...FAGT WKMRSSENFD ELLKALGVNA MLRKVAVAAA SKPHVEIRQD 46
CRABPII  MPN...FSGN WKIIRSENFE ELLKVLGVNV MLRKIAVAAA SKPAVEIKQE 46

 L-FABP  GKHFKFTITA GSKVIQNE.F TVGEECE..L ETMTGEKVKT VVQLEGDNKL 90
 I-FABP  GNKFTVKESS AFRNIEVV.F ELGVTFN..Y NLADGTELRG TWSLEGNKLI 90
 H-FABP  GDILTLKTHS TFKNTEIS.F KLGVEFDE.. TTADDRKVKS IVTLDGGKLV 92
My-FABP  GDIITIRTES TFKNTEIS.F KLGQEFEE.. TTADNRKTKS IVTLQRGSLN 92
 A-FABP  GDVITIKSES TFKNTEIS.F ILGQEFDE.. VTADDRKVKS TITLDGGVLV 92
 E-FABP  GKNLTIKTES TLKTTQFS.C TLGEKFEE.. TTADGRKTQT VCNFTDGALV 94
Il-FABP  GQNFTWSQQY PGGHSITNTF TIGKECD..I ETIGGKKFKA TVQMEG.GKV 90
  CRBPI  GDHMIIRTLS TFRNYIMD.F QVGKEFEEDL TGIDDRKCMT TVSWDGDKL. 93
 CRBPII  GDNFKTKTNS TFRNYDLD.F TVGVEFDEHT KGLDGRNVKT LVTWEGNTLV 94
 CRABPI  GDQFYIKTST TVRTTEIN.F KVGEGFEE.. ETVDGRKCRS LATWENENKI 93
CRABPII  GDTFYIKTST TVRTTEIN.F KVGEEFEE.. QTVDGRPCKS LVKWESENKM 93

 L-FABP  VTTFKNIKSV ......T.EL .NGDIITNTM TLGDIVFKRI SKRI. 126
 I-FABP  ...GKFKRTD NGNELNTVRE IIGDELVQTY VYEGVEAKRI FKKD. 131
 H-FABP  H....LQKWD GQETTLVREL I.DGKLILTL THGTAVCTRT YEKEA 132
My-FABP  Q....VQRWN GKETTIKRKL V.DGKMVAEC KMKGVVCTRI YEKV. 131
 A-FABP  H....VQKWD GKSTTIKRKR E.DDKLVVEC VMKGVTSTRV YERA. 131
 E-FABP  Q....HQEWD GKESTITRKL K.DGKLVVEC VMNNVTCTRI YEKVE 134
Il-FABP  VVNSPNYHH. ......TAEI V.DGKLVEVS TVGGVSYERV SKKLA 127
  CRBPI  ...QCVQKGE KEGRGWT.QW IEGDELHLEM RVEGVVCKQV FKKVQ 134
 CRBPII  C....VQKGE KENRGWK.QW VEGDKLYLEL TCGDQVCRQV FKKK  133
 CRABPI  HCTQTLLEGD GPKTYWTREL AND.ELILTF GADDVVCTRI YVRE. 136
CRABPII  VCEQKLLKGE GPKTSWTREL TNDGELILTM TADDVVCTRV YVRE. 137
```

Fig. 2. Alignment of the amino acid sequences of the members of the FABP family. All sequences are for human proteins, except ileal FABP and CRBP II, respectively for pig and rat. Identical residues present in at least 5 molecules are shaded.

to impede fatty acid binding to adipocyte FABP.[52] On the other hand, fatty acid binding activates phosphorylation of this protein.[124] Insulin receptor tyrosine kinase phosphorylated only less than 0.1% of human muscle FABP in *in vitro* experiments. (Prinsen and Veerkamp, unpublished data). Delipidation or fatty acid loading had no effect. No phosphorylation of FABP was observed with or without insulin stimulation in incubated rat soleus muscle or cultured rat muscle cells. The physiological relevance of tyrosine phosphorylation of FABP is therefore doubtful.

Heterogeneity of protein preparations (immunologically pure) has been found at chromatography and isoelectric focussing for various FABP types, especially for liver FABP.[208,352,374] Many possible causes have been suggested, as fatty acid loading, glutathione-FABP mixed disulfide formation, partial unfolding, N-acetylation, amidation of acidic residues. Recently it was established that amino acid exchange (Asp^{105}–Asn^{105}) and covalent modification by cysteine and glutathione could explain the heterogeneity of FABP from bovine liver.[81] The modifications did not affect fatty acid binding. Only one of two cytoplasmic isoforms of heart FABP was found in mitochondria of bovine heart.[366]

A high degree of similarity exists between the same FABP, CRBP or CRABP type from different mammalian species.[9,374] About the primary structure of members of FABP family in other vertebrates no data are available, although they were also isolated from various tissues of chicken and fish.[374] Muscle FABPs from two locusts, *Schistocerca gregaria*[116,263] and *Locusta migratoria*[193] show 41 and 42% identity of amino acid sequence with human muscle FABP[254] and a high percentage of conservative substitution, especially in residues involved in fatty acid binding.[193] The flatworm, *Schistosoma mansoni* contains a 15 kDa

FJ RESEARCH COMMUNICATIONS

Exhibit III

(III)

Homology and structure-function correlations between α_1-acid glycoprotein and serum retinol-binding protein and its relatives

SYED PERVAIZ AND KEITH BREW

Department of Biochemistry, University of Miami School of Medicine, Miami, Florida 33101, USA

ABSTRACT

Although the serum protein α_1-acid glycoprotein (AGP) or orosomucoid has been extensively studied, its relationships with other proteins have been controversial and its precise physiological function has remained unclear. It is shown here that AGP is significantly similar in amino acid sequence and in the locations of introns in its structural gene to members of a protein superfamily that includes serum retinol-binding protein (RBP), β-lactoglobulin (LG), α_{2u}-globulin, and protein HC (α_1-microglobulin). The view that the three-dimensional structure of AGP is closely similar to the published structures of RBP and LG is supported by its homology with these proteins, similarities in disulfide bond arrangements, and its secondary structure profile, predicted from the amino acid sequence. The relationship of AGP with this particular protein family indicates that its well-characterized ability to bind lipophilic drugs and certain steroids is a reflection of its true biological role. It is proposed that AGP and the other members of this extensive group of proteins should be designated lipocalins to reflect a common ability to bind lipophiles by enclosure within their structures in a manner that minimizes solvent contact. — PERVAIZ, S.; BREW, K. Homology and structure-function correlations between α_1-acid glycoprotein and serum retinol-binding protein and its relatives. *FASEB J.* 1: 209–214; 1987.

Key Words: *α_1-acid glycoprotein · retinol-binding protein · orosomucoid · lipocalin*

α_1-ACID GLYCOPROTEIN (AGP) OR orosomucoid is one of a group of serum proteins (acute phase reactants) whose levels are greatly elevated in inflammatory or stressful states (1). AGP is distinct among serum proteins in its high carbohydrate content (40%) and its highly acidic nature (2, 3), and has been well characterized with respect to its covalent structure, gene structure, and regulation of its expression (2, 4–6). Although AGP has been shown to have various activities that are of possible physiological significance, such as effects on platelet aggregation (7), immunosuppressive activity (8), and the ability to bind a wide range of basic drugs (9–11) as well as steroids such as progesterone (12), it is not clear which of these activities, if any, is a reflection of its biological function.

The realization that a protein is homologous with a group of proteins whose members have known biological functions can often help to identify the nature of its function because, in general, homologous proteins retain some similarity in function at the molecular level. In this regard, it was originally proposed that AGP is related to the immunoglobulin family (2), but later studies revealed that the sequences of AGP and the immunoglobulins do not show a statistically significant correlation (13). In a recent report (14) it has been suggested that a region of sequence of human AGP (residues 21–145) is significantly similar to a region in each of two homologous extracellular domains of the receptor for epidermal growth factor (EGF) (residues 37–169 and 341–485).

Neither of these previously proposed relationships between AGP and other proteins provides any immediate clarification of the function of AGP in vivo, because the activities of the immunoglobulins and the EGF receptor do not appear to be similar to any of the previously mentioned activites of AGP. In addition, analyses described here suggest that there is not a significant correlation between the primary structures of AGP and the EGF receptor. Instead, we show that AGP is similar in amino acid sequence, disulfide bond arrangements, and gene structure to members of a group of proteins that are related to serum retinol-binding protein (RBP). Present information regarding the functions of these proteins indicates that they have a common role in the binding and transport of lipophilic molecules, which suggests that the ability of AGP to bind lipophiles is a reflection of its true physiological function. Based on the known three-dimensional structures of two of these homologs, a general model for the structure of AGP is deduced as well as the nature of its binding site for hydrophobic ligands.

METHODS

Amino acid sequences of proteins, or segments of proteins where appropriate, were aligned and compared by using the computer program ALIGN of the Protein Identification Resource of the National Biomedical Research

0892-6638/87/0001-0209/$01.50.

Foundation (NBRF) (15). The degree of similarity between sequences was evaluated by using the Mutation Data Matrix, with a bias of six and a gap penalty of six (15). The sequences used for comparisons are from the NBRF protein sequence data bank from sources cited in the text. Multiple sequences were aligned by adjusting the pairwise computer alignments to achieve the best overall arrangement, as determined visually (Fig. 1).

RESULTS AND DISCUSSION

Although the comparison of the section of the human AGP sequence (residues 21–145) with segments of the EGF receptor gave alignment scores that were about 3.5 SD from the corresponding scores obtained for randomized sequences of the same amino acid composition, comparisons with the corresponding region of rat AGP (4) gave scores that were insignificant (0.93–1.33 SD from random), whereas comparisons involving the complete sequences of both AGPs and more complete sequences from the two domains of the EGF receptor gave scores that ranged from −0.5 to +1.54 SD from random (only two of eight correlations being positive). The marginal correlation between the region of human AGP (residues 21–145) and the sections of the EGF receptor appears to be fortuitous because the corresponding region of the sequence of rat AGP does not show a significant correlation, and the scores are not significant when complete sequences are used in the comparison. There is also no

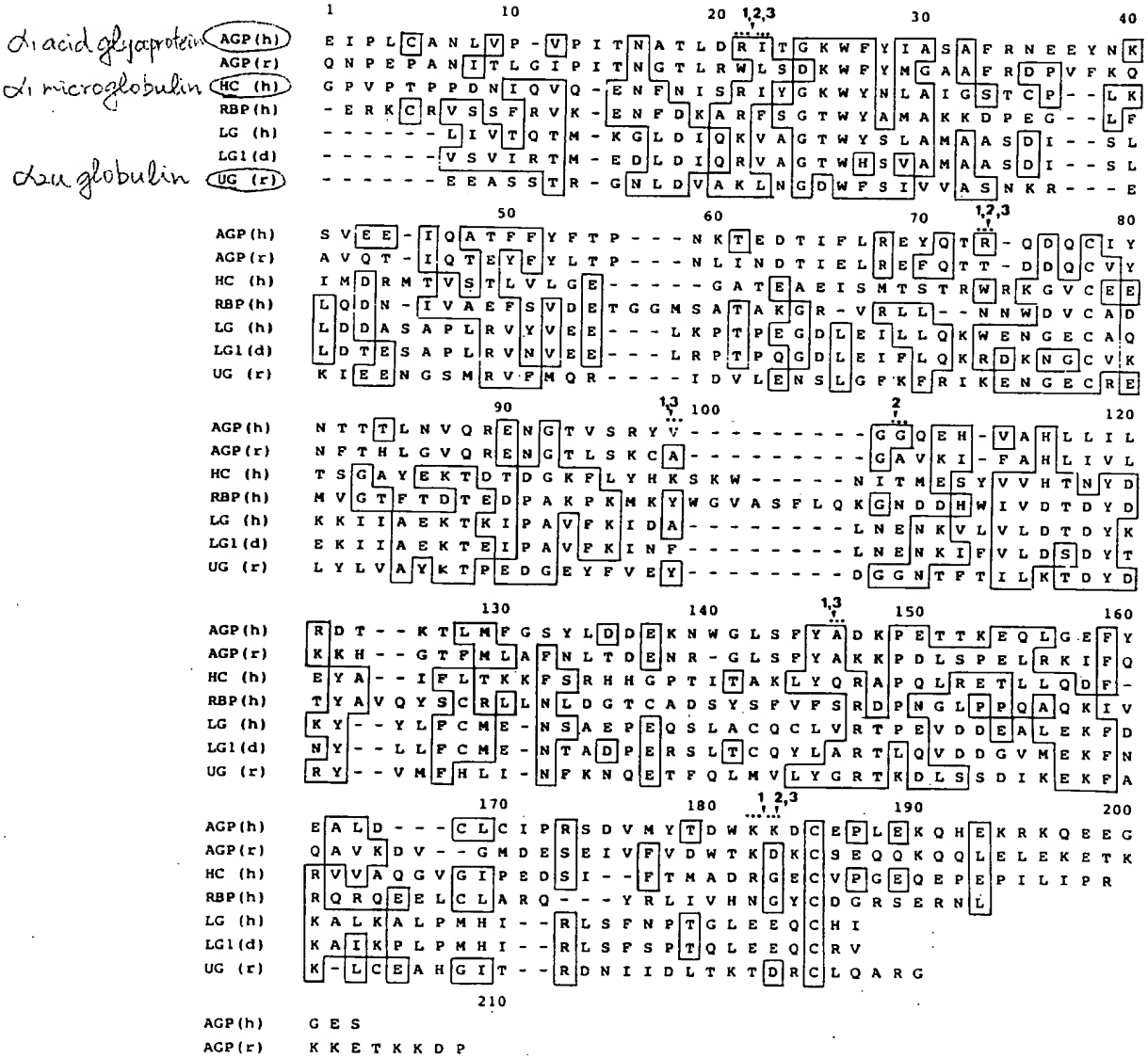

Figure 1. An alignment of the sequences of human and rat AGPs with those of other lipocalins. The sequences were taken from the NBRF protein sequence data bank, apart from that of dolphin LG1 (S. Pervaiz and K. Brew, manuscript in preparation). The arrows indicate the locations of the introns in the genes for: rat AGP, 1; rat RBP, 2; rat α_{2u}-globulin, 3. Filled circles indicate the bases in the codons for the amino acids around the exon/intron boundaries. Residues that are identical in at least two nonisologous proteins are enclosed in boxes.

structural foundation for separating residues 21–145 for comparison with other proteins because they do not represent an intact domain of AGP as reflected by the fact that they contain one-half of a disulfide bond. Furthermore, the major differences between AGP and the EGF receptor in such properties as size, localization (i.e., soluble vs. membrane-bound), and function support the view that these proteins are not related.

COMPARISONS WITH THE RBP SUPERFAMILY

Amino acid sequences

In contrast to the EGF receptor, the members of the superfamily of proteins that are related to serum RBP show similarities to AGP in size and general nature, as well as in structure and function, as discussed below. Besides serum RBP, this family includes the milk protein β-lactoglobulin (LG); the human urine glycoprotein, protein HC or α_1-microglobulin (HC); and a major urine protein from adult male rodents, α_{2u}-globulin or major urinary protein (UG) (16–18).

The results of comparisons of the sequences of both rat and human AGPs with a range of species variants of the proteins in this group provide consistent evidence of relatedness, as summarized in Table 1. For these comparisons, complete sequences were used except for relatively short sections at the amino and/or carboxy termini, regions where differences between homologous proteins are commonly found and that, in the case of these proteins, can be justified on structural grounds (see below). It can be seen that all of these comparisons show positive correlations, five being more than 4.75 SD above random (less than 1 in 10^6 probability of being fortuitous), four between 3.72 and 4.75 SD from random (less than 1 in 10^5), and two between 2.07 and 3.72 SD (less than 1 in 10^3). Both human and rat AGPs showed the strongest correlations with the UGs and the weakest with the RBPs.

Gene structures

The structures of the genes for AGP (5, 6) and two established members of the superfamily UG and RBP (18, 19) have been reported. The genes for AGP and UG were found to consist of six exons separated by five introns, whereas the RBP gene contains five exons and four introns. The locations of the introns are shown in relation to the protein sequences by arrows in the alignment in Fig. 1, where it can be seen that four of the five introns in the AGP gene are located at exactly equivalent sites to introns in either RBP or UG; at two of these sites, all three genes have a similarly placed intron. The last intron in the sequence is located at a corresponding position in RBP and UG, but is shifted by two bases in AGP. In relation to this, is should be noted that the final exon in the sequence is the location of the greatest variability between these proteins (in length) and is also a region that is less ordered in the three-dimensional structures of RBP and LG (20, 21) and does not seem to play an important role in their structures. It is extremely unlikely that the level of similarity of the AGP gene with the RBP and UG genes with respect to exon/intron boundaries could have arisen by chance. Inasmuch as this aspect of gene structure is distinct from primary structure, it can be reasonably concluded, based on this and the sequence correlations discussed previously, that the gene for AGP shares a common ancestry with those for the other proteins in this group.

IMPLICATIONS FOR STRUCTURE AND FUNCTION

Protein-folding patterns are recognized to be highly conserved in homologous proteins even though they may diverge considerably in structure and function. In the case of LG and RBP, a closely similar polypeptide fold is preserved despite a difference of about 80% in the amino acid sequences and despite the necessity of placing several

TABLE 1. *Scores for the alignments of pairs of lipocalins*[a]

	LG (d1)	LG (d2)	LG (b)	LG (ho1)	LG (ho2)	RBP (h)	RBP (r)	RBP (rb)	HC (h)	UG (r)	UG (m)	AGP (h)	AGP (r)
LG (d1)	—	28.0	36.8	31.4	23.8	6.67	6.04	6.40	6.17	9.16	9.42	4.64	3.74
LG (d2)	59.3	—	35.9	29.3	25.0	5.58	5.37	6.39	6.68	10.5	10.8	4.65	3.42
LG (b)	70.4	55.6	—	26.6	32.4	6.44	5.00	6.27	6.75	9.87	8.29	6.05	3.95
LG (ho1)	56.8	49.4	57.4	—	39.0	2.92	2.95	3.42	6.21	9.96	9.54	4.84	3.10
LG (ho2)	50.9	50.0	46.2	70.0	—	2.72	1.85	2.43	3.47	8.14	10.3	2.27	2.98
RBP (h)	23.3	19.5	21.5	21.7	20.8	—	40.4	50.2	7.64	4.98	5.07	1.55	3.95
RBP (r)	21.5	20.7	19.6	20.0	19.6	86.6	—	48.6	6.01	4.74	5.47	1.00	3.49
RBP (rb)	23.3	22.6	21.5	18.9	22.1	91.8	86.8	—	7.28	4.66	5.09	1.43	3.48
HC (h)	18.2	17.5	22.0	25.0	18.5	21.4	18.3	19.6	—	10.1	7.72	4.54	3.72
UG (r)	21.4	22.2	23.9	26.0	21.9	22.2	17.8	20.5	23.4	—	33.2	4.49	6.03
UG (m)	19.6	19.9	18.4	23.1	21.4	19.4	18.4	18.2	21.3	64.2	—	5.51	7.23
AGP (h)	16.9	18.7	18.7	19.0	18.5	18.1	16.8	17.5	20.7	17.4	16.2	—	22.3
AGP (r)	15.6	16.9	16.2	16.1	16.2	18.0	17.6	16.8	15.0	16.0	19.0	45.6	—

[a] The lower half of the matrix gives the percentage of identical residues, and the upper half, the correlation scores in SD units obtained with the program ALIGN, as described in the text. The names of proteins are abbreviated as in the text with their species of origin and isoform indicated in parentheses as follows: d1, dolphin isoform 1; d2, dolphin isoform 2; b, bovine; ho1, horse isoform 1; ho2, horse isoform 2; h, human; r, rat; rb, rabbit; m, mouse.

deletions and/or insertions in their sequences to achieve and optimal sequence alignment (16, 21). Although crystals of AGP have been grown, they were found to be unsuitable for structural analysis above 12 Å resolution (22); in addition, the high carbohydrate content suggests that highly ordered crystals will not be readily prepared. Nevertheless, the homology of AGP with two proteins of known three-dimensional structure suggests that these can be used as basis for a model for the structure of AGP. The presence of a disulfide bond in human and rat AGPs that is also found in RBP and LG (between cysteines 78 and 185 in Fig. 1), and a second that is present in human but not rat AGP, which is similar to one that is found in RBP (between cysteines 5 and 168), provide direct evidence for some similarity in tertiary structure.

In the structures of human RBP and bovine LG, the major part of the polypeptide chain is folded in an eight-stranded β barrel (which encloses the bound retinol) and a single rod of α helix, composed of residues 154–164 in Fig. 1. Application of the Chou and Fasman secondary structure prediction procedure (23) to the amino acid sequence of AGP indicates that it also has a similar fold. Only two regions have a high helix-forming potential: residues 154–164 and 188–200 (numbering based on Fig. 1). The former corresponds exactly to the section of α helix in the RBP and LG structures, whereas the latter represents the carboxy-terminal extension that is not present in these other proteins. In the remaining regions of the AGP sequence, nine sections have high β-structure potential and these are flanked or separated at five sites by potential β-bends. The stretches of β structure, with one exception (positions 7–17), either correspond to or largely overlap sections of the polypeptide chain whose equivalents in LG and RBP form strands of the β barrel. Although model-building studies have not been conducted to fit the AGP sequence into a structure based on the LG and RBP structures, if the predicted secondary patterns are considered in conjunction with the constraints imposed by the known disulfide bond arrangments, it appears that the polypeptide fold of AGP is closely similar to those of LG and RBP.

The region in AGP after the last cysteine in the sequence is highly hydrophilic and appears likely to be solvent-exposed and flexible, like the shorter extension in RBP. As noted previously, it also has a high helix-forming potential and could represent a section of the molecule that is capable of interacting with other molecules. This structure is also compatible with the glycosylation pattern of human AGP because the glycosylated asparagines (at positions 15, 38, 59, 81, and 91) are situated either at β-bends or in regions that have no dominant secondary structure characteristics.

The β barrel fold found in RBP and LG, and presumably in their homologs, in which the interior cavity is lined with largely hydrophobic side chains that can interact favorably with a bound apolar molecular, represents a structure that is well suited to minimizing contacts between a ligand and the surrounding solvent. Although the members of this protein superfamily other than RBP do not have physiological functions that are clearly defined, circumstantial evidence suggests that a common feature is that of binding and transporting lipophilic molecules. Thus, LG appears to function in binding and facilitating the absorption of vitamin A (and possibly other apolar nutrients) from milk (16, 21), HC may carry an unknown lipophile for excretion in urine (16); and UG has been implicated as a possible carrier of a pheromone from the liver to urine in adult male rodents (24). In this context, the well-characterized ability of AGP to bind a wide range of basic drugs with aromatic ring systems, including chlorpromazine as well as steroids such as progesterone, stands out as an activity that reflects its membership in the RBP superfamily and its proposed three-dimensional structure. It is significant that, when isolated from plasma, AGP contains a bound lipophilic ligand or ligands that inhibit the binding of steroids (25), suggesting a role in binding natural lipophiles as well as drugs. Some of the observed activities of AGP, for example on platelets, could be explained as the result of the sequestration of biologically active lipids. The question of whether AGP simply has a physiological role as a scavenger of potentially harmful lipophiles or acts as a carrier of a specific molecule could be clarified by analysis of the native ligand. Although the primary role of AGP appears to be that of a lipid carrier, some of the actions attributed to it may be unrelated to this activity and could be functions of regions of the protein outside the lipid-binding site, including the carbohydrate chains.

Because there are a considerable number of proteins in this family, it seems appropriate that they should be assigned a group name. However, the range of biological contexts and complexities associated with their activities pose a problem in regard to nomenclature. For example, the transport of retinol by RBP involves an association with another protein, transthyretin, in the circulation and the subsequent binding of this complex with cellular receptors, whereas the putative transport activity of UG does not involve binding with other proteins; in the case of AGP, the function may be that of trapping or segregating lipophiles, as opposed to transport. We propose that the proteins of this group should be designated lipocalins to imply a common role in binding lipophilic molecules, and a mode of binding in which the ligand is enclosed by the protein, as is a flower by the calyx. The sequestering of amphiphilic molecules in this type of complex would appear to be important in preventing their unregulated insertion into membranes and other biological structures through random contact.

A similar name might also be applied to the family of intracellular proteins that perform analogous functions in the binding and transport of various lipophiles within the cell. These proteins are cellular RBP, cellular retinoic acid-binding protein, myelin P2 protein, adipocyte 422 protein, and intracellular fatty acid-binding proteins from liver and heart (26, 27). We (16) and others (28) have previously noted a similarity between a region of sequence in LG, RBP, and HC, around residue 26 in Fig. 1, and a section close to the amino terminus of the intracellular group. This is a region where there is some of the strongest conservation of sequence within each of the two families. Outside of this area there is little similarity between the intra- and extracellular proteins, and our

comparisons do not show significant correlations between the complete sequences of individual intracellular proteins and complete or even truncated sequences from the extracellular proteins.

Whether the localized similarity is coincidental or reflects a very distant common ancestry of the genes, or a part of the genes, of the intra- and extracellular groups cannot be resolved at present. For this reason, we suggest that the name lipocalins be reserved for the extracellular proteins discussed here until the question of their relationship with the intracellular group is resolved by crystallographic studies.

The unusually large number of proteins in this family and the diversity of their biological roles emphasize the importance of the segregation and regulated transport of lipophilic molecules in biological systems. Besides the proteins discussed here, three others can be tentatively identified as lipocalins. Schubert and co-workers (29) have shown that purpurin, a component of adherons that promotes cell-adheron adhesion and prolongs the survival of cultured neural epithelial cells, is closely similar to RBP in sequence and can also bind retinol; Lee et al. (30) have reported that a protein encoded by a cDNA from a frog olfactory neuroepithelium cDNA library is homologous with LG, RBP, and HC, and suggest that it may function in binding odorants. Also, we have found significant sequence correlations between insecticyanin (31), a biliverdin transport protein from insect hemolymph, and the same proteins. It is reasonable to speculate that even more lipocalins await discovery. FJ

This work was supported by grant GM 21363 from the National Institutes of Health. We wish to acknowledge use of the facilities of the Protein Identification Resource of the National Biomedical Research Foundation.

REFERENCES

1. Kushner, I. The phenomenon of the acute phase response. *Ann. N.Y. Acad. Sci.* 389: 39-48; 1982.
2. Schmid, K.; Kaufman, H.; Isemura, S.; Bauer, F.; Emura, J.; Motoyarma, T.; Ishiguro, M.; Nanno, S. Structure of α_1-acid glycoprotein. The complete amino acid sequence, multiple amino acid substitutions, and homology with the immunoglobulins. *Biochemistry* 12: 2711-2724; 1973.
3. Schmid, K. Alpha$_1$-acid glycoprotein. Putnam, F., ed. *The plasma proteins: structure, function and genetic control,* vol. I. New York: Academic; 1975: 184-228.
4. Ricca, G. A.; Taylor, J. M. Nucleotide sequence of rat α_1-acid glycoprotein messenger RNA. *J. Biol. Chem.* 256: 11199-11202; 1981.
5. Liao, Y.-C.; Taylor, J. M.; Vannice, J. L.; Clawson, G. A.; Smuckler, E. A. Structure of the rat α_1-acid glycoprotein gene. *Mol. Cell. Biol.* 5: 3634-3639; 1985.
6. Reinke, R.; Feigelson, P. Rat α_1-acid glycoprotein: gene sequence and regulation by glucocorticoids in transfected L-cells. *J. Biol. Chem.* 260: 4397-4403; 1985.
7. Costello, M.; Fiedel, B. A.; Gewurz, H. Inhibition of platelet aggregation by native and desialised α_1-acid glycoprotein. *Nature (London)* 281: 677-678; 1979.
8. Bennett, M.; Schmid, K. Immunosuppression by human plasma α_1-acid glycoprotein. *Proc. Natl. Acad. Sci. USA* 77: 6109-6113; 1980.
9. Piafsky, K. M.; Borga, O.; Odar-Cederlöf, I.; Johansson, C.; Sjoqvist, F. Increased plasma protein binding of propanolol and chlorpromazine mediated by disease-induced elevations of α_1-acid glycoprotein. *N. Engl. J. Med.* 299: 1435-1439; 1978.
10. Denson, D.; Coyle, D.; Thompson, G.; Myers, J. α_1-Acid glycoprotein and albumin in human serum bupivacine binding. *Clin. Pharmacol. Ther.* 35: 409-415; 1984.
11. Friedman, M. L.; Schlueter, K. T.; Kirley, T. L.; Halsall, H. B. Fluorescence quenching of human orosomucoid. Accessibility to drugs and small quenching agents. *Biochem. J.* 232: 863-867; 1985.
12. Kute, T.; Westphal, U. Steroid-protein interaction. XXXIV. Chemical modification of α_1-acid glycoprotein for characterization of the progesterone binding site. *Biochim. Biophys. Acta* 420: 195-213; 1976.
13. Marchalonis, J. J.; Vasta, G. R.; Ware, G. W.; Barker, W. C. Probing the boundaries of the extended immunoglobulin family of recognition molecules: jumping domains, convergence and minigenes. *Immunol. Today* 5: 133-142; 1984.
14. Toh, H.; Hayashida, H.; Kikuno, R.; Yasunaga, T.; Miyata, T. Sequence similarity between EGF receptor and α_1-acid glycoprotein. *Nature (London)* 314: 199; 1985.
15. Dayhoff, M. O.; Barker, W. C.; Hunt, L. T. Establishing homologies in protein sequences. *Methods Enzymol.* 91: 524-545; 1983.
16. Pervaiz, S.; Brew, K. Homology of β-lactoglobulin, serum retinol-binding protein and protein HC. *Science* 228: 335-337; 1985.
17. Unterman, R. D.; Lynch, K. R.; Nakhasi, H. L.; Dolan, K. P.; Hamilton, J. W.; Cohn, D. V.; Feigelson, P. Cloning and sequence of several α_{2u} globulin cDNAs. *Proc. Natl. Acad. Sci. USA* 78: 3478-3482; 1981.
18. Godovac-Zimmermann, J.; Conti, A.; Liberatori, J.; Braunitzer, G. Homology between the primary structures of β-lactoglobulins and human retinol-binding protein: evidence for a similar biological function? *Biol. Chem. Hoppe-Seyler* 366: 431-434; 1985.
19. Laurent, B. C.; Nilsson, M. H. L.; Bavik, C. O.; Jones, T. A.; Sundelin, J.; Peterson, P. A. Characterization of the rat retinol-binding protein gene and comparison to the three-dimensional structure of the protein. *J. Biol. Chem.* 260: 11476-11480; 1985.
20. Newcomer, M. E.; Jones, T. A.; Aqvist, J.; Sundelin, J.; Eriksson, U.; Rask, L.; Peterson, P. A. The three-dimensional structure of retinol-binding protein. *EMBO J.* 3: 1451-1454; 1984.
21. Papiz, M. Z.; Sawyer, L.; Eliopoulos, E. E.; North, A. C. T.; Findlay, J. B. C.; Sivaprasadarao, R.; Jones, T. A.; Newcomer, M. E.; Kraulis, P. J. The structure of β-lactoglobulin and its similarity to plasma retinol-binding protein. *Nature (London)* 324: 383-385; 1986.
22. McPherson, A.; Friedman, M. L.; Halsall, H. B. Crystallization of α_1-acid glycoprotein. *Biochem. Biophys. Res. Commun.* 124: 619-624; 1984.
23. Chou, P. Y.; Fasman, G. D. Empirical predictions of protein conformation. *Annu. Rev. Biochem.* 47: 251-276; 1978.
24. Shaw, P. H.; Held, W. A.; Hastie, N. D. The gene family for major urinary proteins: expression in several secretory tissues of the mouse. *Cell* 32: 755-761; 1983.

25. Ganguly, M.; Carnighan, R. H.; Westphal, U. Steroid-protein interactions XIV. Interaction between human α_1-acid glycoprotein and progesterone. *Biochemistry* 6: 2803–2814; 1967.
26. Sundelin, J.; Anundi, H.; Trägärdh, L.; Eriksson, U.; Lind, P.; Ronne, H.; Peterson, P. A.; Rask, L. The primary structure of rat liver cellular retinol-binding protein. *J. Biol. Chem.* 260: 6488–6493; 1985.
27. Sacchettini, J. C.; Said, B.; Schulz, H.; Gordon, J. I. Rat heart fatty acid-binding protein is highly homologous to the murine adipocyte 422 protein and the P2 protein of peripheral nerve myelin. *J. Biol. Chem.* 261: 8218–8223; 1986.
28. Godovac-Zimmermann, J.; Conti, A.; Liberatori, J.; Braunitzer, G. The amino-acid sequence of β-lactoglobulin II from horse colostrum *(Equus caballus,* Perissodactyla): β-lactoglobulins are retinol-binding proteins. *Biol. Chem. Hoppe-Seyler* 366: 601–608; 1985.
29. Schubert, D.; LaCorbiere, M.; Esch, F. A chick neural retina adhesion and survival molecule is a retinol-binding protein. *J. Cell Biol.* 102: 2295–2301; 1986.
30. Lee, K.-H.; Wells, R. G.; Reed, R. R. Isolation of an olfactory cDNA: similarity to retinol-binding protein suggests a role in olfaction. *Science* 235: 1053–1056; 1987.
31. Riley, C.-T.; Barbeau, B. K.; Keim, P. S.; Kézdy, F. J.; Heinrikson, R. L.; Law, J. H. The covalent structure of insecticyanin, a blue biliprotein from the hemolymph of the tobacco hornworm, *Manduca sexta* L. *J. Biol. Chem.* 259: 13159–13165; 1984.

Received for publication April 5, 1987.
Accepted for publication May 14, 1987.